REMARKS/ARGUMENTS

Status of the Claims:

Claims 1-64 were pending in the present application before this amendment as set forth above. Among them, claims 44-49 and 58-64 were under examination, and claims 1-41 and 50-57 were withdrawn as being drawn to non-elected subject matter. By this Amendment, claim 45 is amended and new claim 65 is added.

The December 12, 2007 Office Action:

In the December 12, 2007 Office Action, claims 45 and 59-64 were rejected under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 4,201,845 to Feder et al. (hereinafter "Feder"). Furthermore, claim 58 was rejected under 35 U.S.C. §103(a) as being unpatentable over Feder.

However, the Primary Examiner indicated that claims 44 and 46-49 were allowed.

The Primary Examiner noted that in the amendment filed September 27, 2007, in response to in the June 27, 2007 Office Action, applicant had amended claim 45 to delete the phrase "for allowing seed biological cells to perfuse only outside the confined region in the chamber". The Primary Examiner further stated that "Examiner placed a telephone call to Applicant's attorney, Tim Xia, to discuss claim 45. Examiner stated that new art [would] be cited against amended claim 45 because it is now broader than the previously submitted claim 45. A discussion of the 112, second paragraph rejection made in the previous Office Action relates to whether the 'biological cells' in the previously submitted claim 42, line 14, from which the previously submitted claim 45 depends, is distinguishable from 'seed biological cells' and clarification as to [its] meaning is requested." Moreover, the Primary Examiner stated that "[a]s stated in the telephone interview, as a courtesy, the present Office action is made non-final to allow Applicant an opportunity to clarify claim 45 if Applicant wishes to reinstate limitations regarding 'seed biological cells.'"

Applicant very much appreciates the Primary Examiner's careful review of the application and the allowance of claims 44 and 46-49, and particularly thanks the Primary Examiner for conducting the telephone interview and the professionalism shown by the Primary Examiner during the telephone interview.

In response, as set forth above, claim 45 has been amended to reinstate the limitation "for allowing seed biological cells to perfuse only inside the confined region in the chamber," which was previously deleted in applicant's September 27, 2007 amendment. Claim 45 has further been amended to correct a typo, i.e., the phase "to perfuse only ***outside*** the confined region in the chamber" has been replaced with the phrase "to perfuse only ***inside*** the confined region in the chamber." (Emphasis added.) Support for this amendment can be found in the disclosure as originally filed, for example, in Fig. 2C.

Furthermore, new claim 65 has been added to recite limitations drawn to the sidewalls of the chamber at the intersection of first and second connection channels with the chamber. Support for this amendment can be found in the disclosure as originally filed, for example, in pages 11 and 26 of the specification and in Figs. 2A1-2F of the drawings.

Moreover, the specification has been amended for better form so that the written description, claims, and drawings are consistent with each other.

Additionally, as discussed in connection with claim 45 below, applicant submits herewith a clarification of the definition of "seed biological cells" as used in the present application, including attached academic articles which clarify the meaning of "seed cells."

Applicant submits that no new matter is added.

Any amendments to the claims not specifically referred to herein as being included for the purpose of distinguishing the claims from cited references are included for the purpose of clarification, consistence, and/or grammatical/spelling correction only.

It is now believed that the application is in condition for allowance and such allowance is respectfully requested.

The following remarks herein are considered to be responsive thereto.

"Seed biological cells" in claim 45:

In the December 12, 2007 Office Action, the Primary Examiner stated that "[a]s stated in the telephone interview, as a courtesy, the present Office [A]ction is made non-final to allow Applicant an opportunity to clarify claim 45 if Applicant wishes to reinstate limitations regarding 'seed biological cells.'" In response, applicant submits that the terms "seeding" and "seed cells" are common usage in the field of bioreactors and known to people skilled in the art, as referring to the cells that are introduced into a previously cell-free bioreactor. These "seed cells" divide

and produce additional cells. This process continues until the bioreactor is filled with cells and either contact inhibition or nutrient limitations constrain further growth and division of cells, or cells are permitted to exit the bioreactor, for example in a chemostat that maintains a constant number of cells in the reactor. In some cases, seed cells are "expanded" from an initial inoculum into a more populous monolayer in a small culture dish or culture flask prior to being transferred into a larger scale bioreactor.

One example of the intended usage of the term "seed cells" is found in "Comparison of manufacturing techniques for adenovirus production," Iyer, P; Ostrove, JM; Vacante, D, Cytotechnology, Volume: 30, Issue: 1-3, Pages: 169-172 (1999), which contains statements such as "Human embryonic kidney (HEK) 293 cells obtained from American Type Culture Collection (ATCC) and grown in fetal bovine serum (Hyclone) containing medium (DMEM, 10% FBS) in 162 cm2 tissue culture flasks were used to ***seed*** Cytodex 3 microcarrier (Pharmacia Biotech) cultures," and "The cells ***seeded*** at 1.5 x 105 c/ml reached 2 x 106 c/ml typically by day 6 or 7 of the culture." (Emphasis added.)

Another example is provided in the article "Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts" by A. Braccini, et al. "Stem Cells Express, published online July 7, 2005; doi:10.1634/stemcells.2005-states: "Here we describe a simple yet innovative bioreactor-based approach to ***seed***, expand and differentiate bone marrow stromal cells (BMSC) directly in a 3D environment, bypassing the conventional process of monolayer (2D) expansion." (Emphasis added.)

The above cited articles for clarifying the meaning of "seed biological cells" are attached following the Remarks/Arguments for the Primary Examiner's reference.

Applicant respectfully submits that the only difference between usages of "seed cells" in these articles cited above and in the application of the present invention is that in the application of the present invention, the term "biological" is inserted in "seed cells" (i.e. to form "seed biological cells") in order to differentiate them from non-biological uses of the term "cells."

Rejections under 35 U.S.C. §102

In the December 12, 2007 Office Action, Claims 45 and 59-64 were rejected under 35 U.S.C. §102(b) as being anticipated by Feder. This rejection is respectfully traversed for at least the following reasons.

Claim 45:

Among other unique limitations, claim 45, as amended, recites a bioreactor comprising:

i. "a first substrate having a first surface and an opposite second surface, defining a chamber therebetween the first surface and the opposite second surface of the first substrate for receiving biological cells and a liquid medium;

ii. an inlet port formed in the first substrate and apart from the chamber;

iii. a first connection channel formed in the first substrate, wherein the first connection channel is in fluid communication with the inlet port and the chamber for allowing a stream of substance to be delivered to the chamber;

iv. an outlet port formed in the first substrate and apart from the chamber;

v. a second connection channel formed in the first substrate, wherein the second connection channel is in fluid communication with the outlet port and the chamber for allowing a stream of substance to be removed from the chamber;

vi. confining means positioned in the chamber to form a confinement region to confine the biological cells therein with the liquid medium,

wherein the chamber, the inlet portion, the first connection channel, the outlet port, and the second connection channel are all formed in the first substrate ***such that, in operation, the stream of substance flows from the inlet port through the first connection channel, the chamber, and the second connection channel to the outlet port in a direction that is substantially parallel to the first substrate***; and wherein the first substrate further defines a first alternate port and a third connection channel in fluid communication with the first alternate port and the confined region of the chamber ***for allowing seed biological cells to perfuse only inside the confined region in the chamber***." (Emphasis added.)

As shown in Figs. 2A1-2I of the drawings as originally filed and described in the specification as originally filed, for example, in paragraphs on line 28-38 of page 18 and lines 7-18 of page 19, the bioreactor according to one embodiment of the present invention is a ***microfluidic*** device which integrates "suitable cell culture and microfabrication techniques to permit cell growth in ***small, confined, well perfused volumes*** at tissue densities, ***provide independent control of*** multiple chemokines and growth factor gradients, ***shear forces, tissue***

perfusion, and permeability of physical barriers to cellular migration, and allow detailed optical and electrochemical observation of normal and cancerous cells during cell migration, intravasation, extravasation, angiogenesis, and other cellular processes." (Emphasis added.)

In contrast, the disclosure of Feder is directed specifically at a class of bioreactor that is radically different from the present invention as claimed in amended claim 45. For instance, Feder states that "[i]n accordance with the present invention, cell culture apparatus for the growth of cells in vitro is provided which employs elongate, selectively permeable hollow fibers in a shallow layer configuration as a matrix for cell attachment on the outer surface of the fibers, and aeration of the cells by passage through the interior of said fibers and permeation of the membrane wall." Feder, Col. 2, lines 22-28. With specific regard to the Primary Examiner's statement that "Part (12) is equivalent to applicant's claimed first substrate," applicant respectfully submits that in Feder, the cells grow on the hollow fibers that are contained within the chamber, and hence, Feder's part 12 is clearly not equivalent to the first substrate of the present invention.

Specifically, as shown in Figs. 2 and 3, Feder discloses a ***macroscopic*** device that is assembles in multiple layers requiring fastening together with bolts and nuts and pipe. In operation of Feder's device, "cell culture medium is ***fed into chamber 23 through inlet ports 26 and 27***. The medium is inoculated through port 29 with a seed culture …. During the incubation, periodic changes of media can be made, with the spent medium ***being expelled through outlet port 28*** and fresh medium again being supplied through inlet ports 26 and 27. Samples of macromolecular materials can be ***withdrawn through access port 29*** at any desired time during the incubation. ***The culture medium flows into the lower part of chamber 23 beneath perforated plate 35 which thereby serves as manifold or distributor means*** to provide uniform distribution of the medium and ***a flow path which is upward and transverse to the plane of the elongate axes of the fibers***. The reduction of the depth of the upper part of chamber 23 in a manner dependent upon its distance from the media outlet 28 assists in the uniform collection of the spent media across the top of the bed of fibers in a manner which corresponds to the demand for the media passing across the fibers." (Feder, col. 4, lines 56-68 through col. 5, lines 1-29, and Figs. 2 and 3) (Emphasis added.) In other words, in Feder's device, ***the culture medium flows from the inlet ports 26 and 27, through the perforated plate 35 into the fiber***

layer 34, and then is expelled through outlet port 28, i.e., the flow of the culture medium is transverse to the planar region containing the cells, i.e. the bed of parallel hollow fibers.

Therefore, applicant respectfully submits that Feder neither teaches nor suggests a microfabricated bioreactor having "the chamber, the inlet portion, the first connection channel, the outlet port, and the second connection channel all formed in the first substrate, ***such that, in operation, the stream of substance flows from the inlet port through the first connection channel, the chamber and the second connection channel to the outlet port in a direction that is substantially parallel to the plane of the first substrate"*** as recited in amended claim 45 of the present invention. (Emphasis added.)

For at least these reasons, amended claim 45 is patentable under 35 U.S.C. §102(b) over Feder.

Accordingly, previously presented claims 58-64, which depend from now allowable amended claim 45, are also allowable for at least this reason.

Additionally, claims 58-64 also contain additional patentable subject matter. For example, claim 60 recites a bioreactor having all the limitations of claim 59 and also comprising "at least one supporting member outside the confined region of the chamber for supporting the second substrate." In contrast, the device of Feder, as shown in Fig. 2, uses element 37, which is a bolt or a stud, as evidenced by the wingnut 22. Thus, Feder does not teach or suggest a bioreactor having all the limitations of claim 60.

Furthermore, claim 61 also contains additional patentable subject matter, and recites a bioreactor having all the limitations of claim 59 and "further comprising at least one supporting member positioned inside the confined region of the chamber for supporting the second substrate." This is shown as central post 1087 in Figs. 2C and 2E, and as posts 1087 in Figs. 2D and 2F of the drawings as originally filed. In contrast, the lip inside of element 12 in Feder is not for the purpose of supporting the second substrate (20), but to hold in position the layer of fibers (34) and the distributor plate (35). Moreover, the second substrate in Feder is held against the first substrate (15) by the bolt (37) and nut (22). Thus, Feder does not teach or suggest a bioreactor having all the limitations of claim 61.

Still further, claim 62 contains additional patentable subject matter, reciting a bioreactor having all the limitations of claim 45 and wherein the stream of substance is controlled as to

provide a gradient to the channel. As shown in Fig. 2A1 of the present invention, the inlet channels 1011a, 1021a, and 1022a provide for three separate fluidic streams that, when merged in the extension of 1021a, will deliver three parallel streams to the chamber 1006. Because laminar flow is maintained by the chosen shape of the channels, chemical gradients can be maintained transverse to the flow to allow cells in different regions in the chamber to be exposed to different concentrations. In contrast, the device of Feder is not capable of supporting laminar flow, and hence there can be turbulence or other mixing in the inlet area beneath the distributor plate (e.g. element 35, Fig. 2). Thus, Feder does not teach or suggest a bioreactor having all the limitations of claim 62.

Rejections under 35 U.S.C. §103

Claim 58:

In the December 12, 2007 Office Action, claim 58 was rejected under 35 U.S.C. §103(a) as being unpatentable over Feder.

As set forth above, claim 58 depends from now allowable claim 45, and should be allowable at least for this reason.

New Claim 65:

New claim 65 depends from now allowable amended claim 45 and should be allowable as well, for at least this reason.

Moreover, new Claim 65 contains also additional patentable subject matter. It recites a bioreactor having all the limitations of independent claim 45, and further limitations: "***sidewalls at the intersections of the first connection channel with the chamber and the second connection channel with the chamber are tapered to form an angle of inclination between about 10 and 45 degrees from vertical and an enclosed angle between about 30 and 80 degrees.***" As shown in Fig. 2C and set forth in lines 1-10 of page 26 of the specification, as originally filed, "***sidewalls of the chamber 1006 are tapered at the intersections of the connection channels with the chamber 1006 to form an angle of inclination α, which is preferred in the range of about between 10 - 45° from vertical, and an enclosed angle β, which is preferred in the range of about between 30 - 80°, respectively, to avoid shear forces generated by sharp corners***." (Emphasis added.) This allows for the utilization of laminar flow

in microfluidics to ensure uniform perfusion of the cells, ***where the fluid flow is parallel to a planar layer of cells grown on the first substrate.***

In contrast, as understood by Applicant, Feder discloses a macroscopic device that is assembled with nuts and bolts (see e.g. Feder, col. 3, lines 13-18). It "employs elongate, selectively permeable hollow fibers in a shallow layer configuration as a matrix for cell attachment on the outer surface of the fibers, and aeration of the cells by passage through the interior of said fibers and permeation of the membrane wall." The flow path of the culture media is directed by a distributor plate means (e.g. element 35 in Figs. 1-3) or a filter plate (e.g. element 40 in Fig. 4) through the fiber layer and substantially transverse to the planar region containing the cells. Feder, Col. 2, lines 22-41 and Figs. 1-4. ***Feder relies solely on the distributor plate 35 or the filter plate 40 to distribute the incoming perfusate through hydraulic resistance, and does not otherwise control the flow or distribution of the incoming flows***.

In other words, Feder does not teach or suggest a bioreactor "***wherein sidewalls at the intersections of the first connection channel with the chamber and second connection channel with the chamber are tapered to form an angle of inclination between about 10 and 45 degrees from vertical and an enclosed angle between about 30 and 80 degrees,***" as recited in new claim 65.

CONCLUSION

Applicant respectfully submits that the foregoing Amendment and Response place this application in condition for allowance. If the Primary Examiner believes that there are any issues that can be resolved by a telephone conference, or that there are any informalities that can be corrected by an Primary Examiner's amendment, please call the undersigned at 404.495.3678.

Respectfully submitted,
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March 12, 2008

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Comparison of manufacturing techniques[logo] for adenovirus production

Authors: Iyer, Paddy; Ostrove, Jeffrey; Vacante, Dominick
Source: Cytotechnology, Volume 30, Numbers 1-3, July 1999 , pp. 169-172(4)
Publisher: Springer

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Abstract:

We have compared three different production methods, which may be suitable for the large scale production of adenovirus vectors for human clinical trials. The procedures compared 293 cells adapted to suspension growth in serum-free medium in a stirred tank bioreactor, 293 cells on microcarriers in serum-containing medium in a stirred tank bioreactor, and 293 cells grown in standard tissue culture plasticware. With a given virus, yields varied between 2000 and 10,000 infectious units/cell. The stirred tank bioreactor routinely produced between 4000 and 7000 infectious units/cell when 293 cells were grown on microcarriers. The 293 cells adapted to suspension growth in serum-free medium in the same stirred tank bioreactor yielded between 2000 and 7000 infectious units/cell. Yields obtained from standard tissue culture plasticware were up to 10,000 infectious units/cell. Cell culture conditions were monitored for glucose consumption, lactate production, and ammonia accumulation. Glucose consumption and lactate accumulation correlated well with the cell growth parameters. Ammonia production does not appear to be significant. Based on virus yields, ease of operation and linear scalability, large-scale adenovirus production seems feasible using 293 cells (adapted to suspension/serum free medium or on microcarriers in serum containing medium) in a stirred tank bioreactor.

Keywords: adenovirus; bioreactor; microcarriers; serum-free medium; 293 cells
Document Type: Research article
DOI: 10.1023/A:1008040221630

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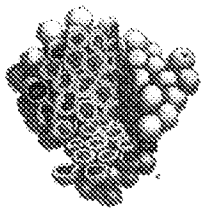

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Three-Dimensional Perfusion Culture of Human Bone Marrow Cells and Generation of Osteoinductive Grafts

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Three-Dimensional Perfusion Culture of Human Bone Marrow Cells and Generation of Osteoinductive Grafts

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Key Words. Bone marrow stromal cells • Bone marrow cells • Colony formation • Expansion Hematopoiesis • Mesenchymal stem cells • Osteoprogenitor • Tissue regeneration

ABSTRACT

Three-dimensional (3D) culture systems are critical to investigate cell physiology and to engineer tissue grafts. In this study, we describe a simple yet innovative bioreactor-based approach to seed, expand, and differentiate bone marrow stromal cells (BMSCs) directly in a 3D environment, bypassing the conventional process of monolayer (two-dimensional [2D]) expansion. The system, based on the perfusion of bone marrow–nucleated cells through porous 3D scaffolds, supported the formation of stromal-like tissues, where BMSCs could be cocultured with hematopoietic progenitor cells in proportions dependent on the specific medium supplements. The resulting engineered constructs, when implanted ectopically in nude mice, generated bone tissue more reproducibly, uniformly, and extensively than scaffolds loaded with 2D-expanded BMSCs. The developed system may thus be used as a 3D in vitro model of bone marrow to study interactions between BMSCs and hematopoietic cells as well as to streamline manufacture of osteoinductive grafts in the context of regenerative medicine. *STEM CELLS 2005;23:1066–1072*

INTRODUCTION

Bone marrow stromal cells (BMSCs) have received increasing experimental and clinical interest, owing to their surprising degree of plasticity [1–3] and their potential use for treatment of genetic [4] or immunologic [5] pathologies. In the field of regenerative medicine, BMSCs have been most extensively used for bone repair because their default pathway seems to be osteogenic [6]. This has led to encouraging findings in heterotopic models [7, 8], in orthotopic implants [9, 10], and in a few clinical cases [11]. Given their low frequency among bone marrow–nucleated cells (approximately 0.01%), BMSCs are typically selected and expanded by sequential passages in monolayer (two-dimensional [2D]) cultures. However, 2D-expanded BMSCs have a dramatically reduced differentiation capacity compared with those found in fresh bone marrow [12, 13], which limits their potential use for therapeutic purposes [6, 14].

Reasoning that a three-dimensional (3D) culture system may represent a more physiological environment than a Petri dish for a variety of cells [15, 16] and that fluid flow is an important component for seeding and culturing BMSCs in 3D environments [17, 18], we aimed in this work at developing an innovative procedure to seed and expand BMSCs directly into porous 3D scaffolds under perfusion. We demonstrated that perfusion of bone marrow–nucleated cells through the pores of 3D ceramic scaffolds resulted in the efficient expansion of clonogenic BMSCs and in the generation of highly osteoinductive grafts. Moreover, the developed system allowed us to coculture BMSCs with hematopoietic cells and to support hematopoiesis.

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Materials and Methods

Bone Marrow Cell Culture

Bone Marrow Aspirates

Bone marrow aspirates (20- to 40-ml volumes) were obtained from eight healthy donors (36–54 years old) during routine orthopedic surgical procedures in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Nucleated cells were isolated from aspirates by Ficoll density-gradient centrifugation. The initial number of BMSCs, defined as the number of fibroblast colony-forming units (CFU-F) in the fresh marrow aspirates, averaged 21 ± 7 per 10^5 nucleated cells.

Culture Medium

Unless otherwise stated, medium (α-modified Eagle's medium) containing 10% fetal bovine serum was supplemented with 5 ng/ml fibroblast growth factor-2, 10 nM dexamethasone, and 0.1 mM L-ascorbic acid-2-phosphate to increase BMSC proliferation and osteogenic commitment [8, 19]. In some experiments, medium was alternatively supplemented with 2 ng/ml interleukin-3, 10 ng/ml stem cell factor, and 20 ng/ml platelet-derived growth factor-bb to support maintenance of hematopoietic cells in culture [20] (hematopoietic medium).

3D Culture

Using a perfusion bioreactor system we previously developed for cell seeding of 3D scaffolds [18], an average of 18.4 ± 6.6 million freshly isolated bone marrow–nucleated cells were perfused through 8-mm-diameter, 4-mm-thick disks of porous (total porosity, 83% ± 3%; pore size distribution: 22%, <100 μm; 32%, 100–200 μm; 40%, 200–500 μm; 6%, >500 μm) hydroxyapatite ceramic (Engipore; Fin-Ceramica Faenza, Faenza, Italy, http://www.fin-ceramicafaenza.com) at a superficial velocity of 400 μm per second (previously determined to result in efficient and uniform cell seeding). Based on CFU-F assays of five marrow aspirates, an estimated average of $4.8 \pm 2.6 \times 10^5$ BMSCs was perfused through each disk, corresponding to 4 BMSCs per cm^2 of ceramic surface area. Such clonogenic BMSC seeding density was previously described to prolong BMSC lifespan and differentiation potential [14]. After 5 days (cell seeding phase), harvested medium was plated in tissue culture dishes to quantify the fraction of CFU-F not seeded. Fresh medium was then added to the system, and the cell-ceramic constructs were perfused for an additional 14 days (cell expansion phase) at a velocity of 100 μm per second (previously determined to support cell viability throughout the scaffold thickness), with medium changes twice a week. As a control, bone marrow–nucleated cells from each donor were plated on tissue-culture dishes (2D expansion) using the same initial cell number/surface area as in the 3D ceramic disks and cultured for 19 days without passaging, with the same schedule of medium changes as for the 3D culture.

Bone Formation Assays

Construct Implantation

Constructs from four independent experiments, after the cell seeding or cell expansion phases of 3D culture, were implanted ectopically in recipient nude mice (CD-1 nu/nu, 1 month old; Charles River Laboratories, Sulzfeld, Germany, http://www.criver.com/index.html) in accordance with institutional guidelines. As a control, we implanted ceramics seeded with 2D-expanded BMSCs at the same density as measured in the corresponding 3D cultured constructs after the cell expansion phase. Seeding of 2D-expanded BMSCs was performed by static loading of a cell suspension. We previously reported that the fraction of cells retained in the scaffolds after seeding by static loading was similar to that obtained using the described perfusion device, although cells seeded statically were less uniformly distributed [18].

Quantitative Assessment of Bone Tissue Formation

Eight weeks after implantation, constructs were fixed in 4% formalin, decalcified (Osteodec; Bio-Optica, Milan, Italy, http://www.bio-optica.it), paraffin embedded, and sectioned at six different levels (5-μm-thick sections at 600-μm intervals). For each cross-section, stained by hematoxilin/eosin, six images (covering most of the total cross-sectional area) were used to quantify the amount of bone tissue normalized to the total available pore space, as previously described [21]. The uniformity of bone tissue formation was quantitatively determined from the average ($\bar{x}$) and standard deviation (s) of the bone amounts measured in each cross-section [18] as follows:

$$\%\ uniformity = 100 \times \left(1 - \left(\frac{s}{\bar{x}}\right)\right)$$

Cell Characterization

Scanning Electron Microscopy

Constructs cultured in the 3D system after the cell expansion phase were fixed in 4% formalin, dehydrated, critical point dried, and coated with 20 nm of Au. Scanning electron microscopy observation was performed with an ESEM XL 30 (Philips, Amsterdam, The Netherlands, http://www.philips.com) with 10-kV acceleration.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

mRNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), treated with DNAse, and retrotranscribed into cDNA, as previously described [19]. Polymerase chain reaction was performed and monitored with the ABI Prism 7700 Sequence Detection System (PerkinElmer/Applied Biosystems, Rotkreuz, Switzerland, http://www.perkinelmer.com), and

expression levels of genes of interest (bone sialoprotein [BSP], collagen type I [CI], and osteopontin [OP]) were normalized to the 18S rRNA. Previously determined levels of expression of the genes of interest in human osteoblast cultures, also normalized to 18S rRNA [19], were used as reference.

Cell Extraction

After the cell expansion phase in the 3D culture system, cells were extracted from the ceramic pores by perfusing a solution of 0.3% collagenase and 0.05% trypsin/0.53 mM EDTA at 400 μm per second. Extracted cells were assessed for the ability to form fibroblastic and hematopoietic colonies and characterized by flow cytometry, as described below.

CFU-F Assay

CFU-F assays of expanded cells were performed by plating four cells per cm^2 in tissue culture dishes. After 10 days of culture, cells were fixed in 4% formalin and stained with 1% methylene blue, and the number of colonies was counted.

Hematopoietic Colony-Forming Unit Assay

Hematopoietic colony-forming unit assays were performed as previously described [22] to quantify the following types of hematopoietic clonogenic cells: neutrophils, macrophages, burst-forming-unit-erythroid, and granulocyte-erythroblast-macrophage-megakariocyte. Briefly, 2.5×10^5 cells per ml were cultured in medium containing 1.75 U/ml erythropoietin, 2.625 ng/ml granulocyte-colony stimulating factor, 40 U/ml granulocyte macrophage colony stimulating factor, 40 U/ml interleukin-3, and 62.5 ng/ml stem cell factor. After 14 days, the colonies were classified and counted.

Fluorescence-Activated Cell Sorting Analysis

Cell suspensions were incubated with antibodies against CD105 (Serotec), STRO-1, BSP, CI, OP (all from Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), nerve growth factor receptor (NGFR), or CD45 (both from Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and analyzed using a FACSCalibur flow cytometer (Becton, Dickinson and Company). Reactions with anti-BSP, -OP, or -CI were proceeded by membrane permeabilization with BD Cytofix/Cytoperm Plus Kit (Becton, Dickinson and Company). Positive expression was defined as the level of fluorescence greater than 95% of corresponding isotype-matched control antibodies.

Results and Discussion

BMSC Expansion Under 3D Perfusion

Using a bioreactor system recently developed for efficient and uniform seeding of anchorage-dependent cells into 3D scaffolds [18], we perfused the nucleated cells of human bone marrow aspirates in alternate directions through the pores of disk-shaped ceramic scaffolds, and we hypothesized that BMSCs would attach to the ceramic substrate and proliferate. The number of BMSCs perfused through each scaffold, estimated by CFU-F assays, averaged $4.8 \pm 2.6 \times 10^5$ cells. Medium was first changed after 5 days (cell seeding phase), which resulted in the elimination of the non-attached cell population, containing negligible numbers of CFU-F (<1% of those seeded in the scaffolds). Fresh medium was further perfused for an additional 14 days (cell expansion phase), during which time the total number of cells, monitored by Alamar blue, was found to increase at a nearly exponential rate (Fig. 1). At 19 days, the number of BMSCs found within the ceramic pores, calculated as the $CD105^+$ fraction of the extracted cells, averaged $9 \pm 3 \times 10^5$ cells for each scaffold. These data demonstrate that BMSCs can be seeded and extensively expanded (average of 8.2 ± 0.9 doublings in 19 days) by perfusion of bone marrow cell suspensions through 3D porous scaffolds, thereby avoiding typical 2D expansion.

Bone Formation by Expanded BMSCs

The osteoinductivity of the constructs resulting from BMSC seeding and expansion in the porous ceramic under perfusion (total of 19 days culture) was verified by ectopic implantation in nude mice. Reproducible, extensive, and markedly uniform bone formation was found in implanted constructs from four out of four independent experiments, performed using aspirates from different donors. Mature lamellar bone, organized in typical bone/marrow ossicles [23], filled an average of 52.1% ± 7.7% of the total available pore space and was distributed throughout the scaffold volume with high uniformity (Fig. 2). In contrast,

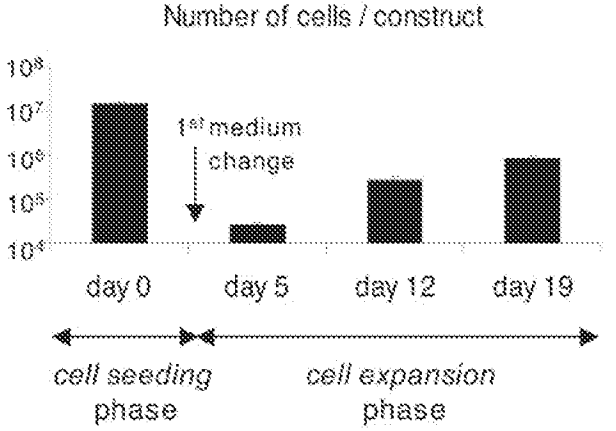

Figure 1. Total number of cells per construct detected in the three-dimensional (3D) system by Alamar blue assays. At day 0, the number of cells corresponds to the total number of cells added to the 3D system. At day 5, after removing the non-adherent cells with the first medium change, the total number of cells corresponds to the cells attached to the scaffold.

when 2D-expanded BMSCs from the same donors were loaded into ceramic scaffolds at the same density as measured in the corresponding 3D cultured constructs, bone tissue was formed in only one of the four experiments. Moreover, in those constructs positive for bone formation, bone tissue filled only 9.6% ± 2.7% of the total available pore space and was localized to scattered peripheral regions (Fig. 2). The increased osteoinductivity of constructs generated using the developed system may have been supported by the ceramic substrate used for BMSC expansion [24], the 3D cell–cell interactions during culture [25], the regimen of fluid flow applied [17, 26], or combinations of these variables that remain to be further elucidated. Interestingly, constructs implanted immediately after the cell seeding phase, in which BMSCs were attached to the ceramic but had not significantly expanded, were never osteoinductive. This suggests that a critical density of osteoprogenitor cells is necessary to initiate bone formation and points out the limit of approaches based on direct implantation of scaffolds mixed with bone marrow aspirates, especially considering the known variability in the number of BMSCs per aspirate volume [27].

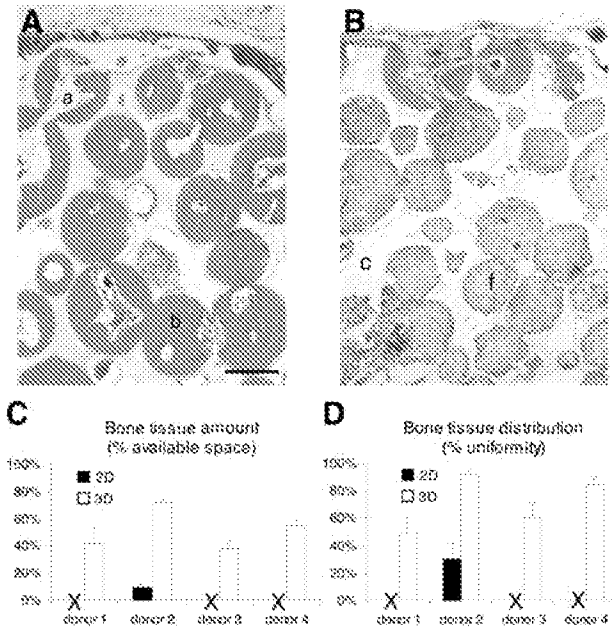

Figure 2. Bone tissue formation by bone marrow stromal cells (BMSCs) expanded in monolayers (two-dimensional [2D]) or under three-dimensional (3D) perfusion. **(A, B):** Representative hematoxilin/eosin-stained cross-sections of BMSC-ceramic constructs implanted ectopically in nude mice and harvested after 8 weeks. BMSCs expanded directly in the ceramic scaffolds in the 3D system yielded massive and uniformly distributed bone tissue **(A)**, in contrast to BMSCs loaded in the ceramic after traditional 2D culture **(B)**. White spaces correspond to the decalcified ceramic (c), whereas scaffold pores are filled with fibrous (f), adipose (a), or bone (b) tissue. Bar = 400 μm. **(C, D):** Quantitative image analysis of constructs generated using bone marrow aspirates from four independent donors further highlighted the increased reproducibility, amount **(C)**, and uniformity **(D)** of bone tissue formation after BMSC expansion under 3D compared with 2D. Values are presented as mean and SE of the percentages calculated for each cross-section. The crosses indicate no bone formation in any of the implanted constructs.

BMSC Characterization

We then preliminarily characterized the morphology, phenotype, and clonogenicity of cells seeded and expanded within the developed 3D system. Scanning electron microscopy indicated the formation of a stromal-like tissue within the ceramic pores, consisting of a 3D network of spheroidal cells in contact with heterogeneously shaped fibroblastic cells (Fig. 3A). The mRNA expression levels of genes encoding for the osteoblast-related proteins BSP, CI, and OP averaged, respectively, 3.6%, 35.3%, and 48.0% of those previously quantified in human osteoblast cultures [19] (Fig. 3B). Levels were similar to those measured in 2D-expanded BMSCs and lower than those measured in BMSCs after osteogenic differentiation [19]. Fluorescence-activated cell sorting analyses indicated that 68% ± 18% of the cells extracted from the ceramic scaffolds were positive for CD105, a surface marker typically expressed by cells of the mesenchymal lineage (Fig. 3C). These CD105$^+$ cells expressed low levels of STRO-1

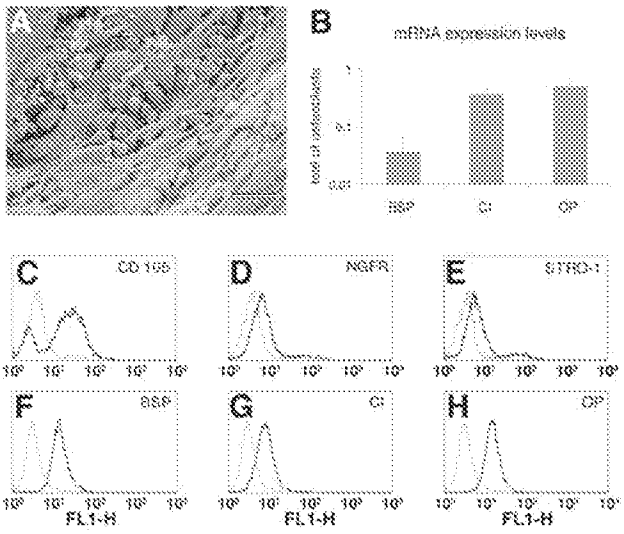

Figure 3. Morphology and phenotype of cells expanded under three-dimensional (3D) perfusion. **(A):** Scanning electron microscopy images of the constructs generated by perfusion of bone marrow–nucleated cells through the pores of ceramic scaffolds for 19 days. The ceramic pores were filled with a stromal-like tissue, consisting of a 3D network of heterogeneously shaped cells and extracellular matrix. Bar = 10 μm. **(B):** mRNA expression levels of bone sialoprotein (BSP), collagen type I (CI), and osteopontin (OP) in the cells. Values are presented as mean and SE of three independent experiments. **(C–H):** Surface markers expressed by cells extracted from the ceramic scaffolds after 19 days culture. Cells positive for **(C)** CD105 expressed low levels of **(D)** nerve growth factor receptor (NGFR) and **(E)** STRO-1 and high levels of **(F)** BSP, **(G)** CI, and **(H)** OP. Light line, isotype control; dark line, specific antibody.

(proposed as a marker of early mesenchymal progenitors [28]) and NGFR (proposed as a marker of multipotent BMSCs [29, 30]) and high levels of BSP, OP, and CI (Figs. 3D–3H). The percentage of CD105$^+$ cells capable of forming a fibroblastic colony (CFU-F) was markedly higher after expansion in the 3D than in typical 2D cultures (29.4% vs. 10.7%, respectively). Taken together, these data suggest that BMSCs generated in the developed 3D system were neither early undifferentiated mesenchymal precursors nor fully differentiated osteoblast-like cells but comprised a large population of clonogenic osteoprogenitor cells. Future studies should address whether changes in the substrate used (e.g., scaffold composition or architecture), flow rate, and culture medium composition will regulate the phenotype, proliferation, and multilineage differentiation capacity of the expanded BMSCs.

Hematopoietic Cell Characterization

The finding that a substantial fraction of the cells cultured in the developed 3D system was not of the mesenchymal lineage, as suggested by the rounded morphology and demonstrated by the lack of expression of CD105, induced us to investigate whether both hematopoietic and mesenchymal cells were cocultured within the ceramic pores. Indeed, in the engineered constructs we found cells positive for CD45, a surface marker of hematopoietic cells, at percentages (30% ± 15%) equivalent to those of cells negative for CD105 (Figs. 4A–4I). It is likely that cocultured hematopoietic cells, possibly including CD14-positive adherent macrophages, regulated the phenotype of BMSCs [31] and played a critical role in determining the osteoinductivity of the constructs, possibly by maintaining a higher fraction of clonogenic BMSCs. It has been described that upon transplantation into a host animal, BMSCs form an ectopic ossicle in which bone cells, myelosupportive stroma, and adipocytes are of donor origin whereas hematopoiesis and the vasculature are of recipient origin [23]. Considering that in our 3D system human hematopoietic cells were coimplanted with BMSCs, future studies should aim at determining whether human cells contributed to hematopoiesis in this model.

We next hypothesized that, through the addition of specific medium supplements, the developed 3D culture model allows the regulation of the relative proportions of hematopoietic and mesenchymal cells. Using supplements typically used for culture of hematopoietic cells (i.e., interleukin-3, stem cell factor, and platelet-derived growth factor-bb, hematopoietic medium) [20], the fraction of CD45$^+$ cells found after 19 days of 3D culture was increased to more than 90% (Fig. 4I) whereas BMSC proliferation capacity was still sustained (average of 4.5 ± 0.7 doublings in 19 days). Interestingly, the use of this culture medium further increased the percentage of CFU-F within CD105$^+$ cells from 29.4%–38.8% and generated relevant fractions of hematopoietic CFUs, including those with a mixed phenotype, indicative of early multilineage progenitor populations (Fig. 4J). Remarkably, the use of the same medium supplements in 2D cultures was not able to modulate the fractions of hematopoietic/mesenchymal cells nor their clonogenicity, possibly due to the fact that most of the non-adherent cells were not entrapped within the 3D niches of the ceramic or newly formed stromal-like tissue and were thus discarded during medium changes. This evidence further highlights the potential of the developed culture system, in which the 3D configuration under perfusion flow provides an extension of the concept of stromal feeder layer for the support and development of hematopoietic cells [23, 32] and thus modifies standard paradigms for culture of bone marrow cells.

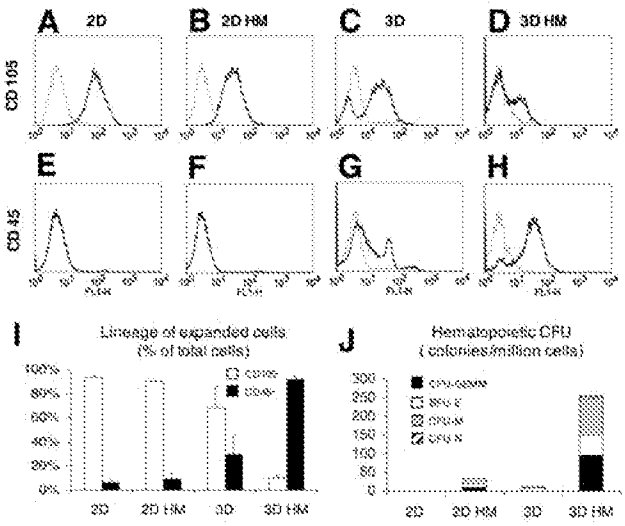

Figure 4. Fraction and clonogenicity of hematopoietic cells. (A–H): Representative profiles of cells labeled for CD105 or CD45 after two-dimensional (2D) or three-dimensional (3D) culture in standard or hematopoietic medium (HM). Light line, isotype control; dark line, specific antibody. (I): Percentages of CD105$^+$ and CD45$^+$ cells in the above conditions. Values are presented as mean and SE of four independent experiments. (J): Quantification of the following types of hematopoietic colony-forming units present within the populations generated in the above conditions: neutrophils (CFU-N), macrophages (CFU-M), burst-forming-unit-erythroid (BFU-E), and granulocyte-erythroblast-macrophage-megakaryocyte (CFU-GEMM).

Conclusions

Our study validates the simple but innovative concept that BMSCs can be seeded and expanded by perfusion culture through the pores of 3D scaffolds starting from minimally processed bone marrow aspirates and avoiding 2D culture expansion. The developed approach was used for the reproducible, spatially uniform, highly efficient, and simplified manufacture of osteoinductive grafts. Incorporating in the system features like automated medium change, monitoring and control of pH, gases, and metabolites are likely to lead to the development of a closed system for the automated and controlled production of autologous BMSC-based bone substitutes. Compared with previously proposed perfusion systems [17, 33], the elimination of the 2D culture would allow for

a one-phase, streamlined procedure that could thus generate engineered bone grafts at reduced costs and make them commercially viable against alternative off-the-shelf osteoinductive materials (e.g., based on the delivery of growth factors). In this context, however, scaling up of the procedure to clinically relevant sizes will have to address the challenge of maintaining cell viability in larger constructs, both during in vitro culture and upon grafting.

Beyond the relevance in the field of bone tissue engineering, our results validate the developed process as a first step toward ex vivo tissue engineering of bone marrow as a model to investigate proliferation, differentiation, and interactions among different types of bone marrow cells in a more physiological environment than previously established systems (e.g., Petri dishes or spinner flasks [20]). The developed culture system may be further explored for the expansion under perfusion of $CD34^+$ hematopoietic stem cells from bone marrow or cord blood within an engineered 3D stromal network. Finally, the same paradigm of bypassing 2D expansion by direct 3D perfusion culture may be used for the engineering of other 3D tissues and organs.

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References

1 Bianco P, Gehron RP. Marrow stromal stem cells. J Clin Invest 2000;105:1663–1668.

2 Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A 2001;98:7841–7845.

3 Jiang Y, Jahagirdar BN, Reinhardt RL et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41–49.

4 Horwitz EM, Prockop DJ, Fitzpatrick L A et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309–313.

5 Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439–1441.

6 Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 2004;32:414–425.

7 Haynesworth SE, Goshima J, Goldberg VM et al. Characterization of cells with osteogenic potential from human marrow. Bone 1992;13:81–88.

8 Muraglia A, Martin I, Cancedda R et al. A nude mouse model for human bone formation in unloaded conditions. Bone 1998;22:131S–134S.

9 Kon E, Muraglia A, Corsi A et al. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000;49:328–337.

10 Petite H, Viateau V, Bensaid W et al. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18:959–963.

11 Quarto R, Mastrogiacomo M, Cancedda R et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385–386.

12 Banfi A, Muraglia A, Dozin B et al. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp Hematol 2000;28:707–715.

13 Mendes SC, Tibbe JM, Veenhof M et al. Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. Tissue Eng 2002;8:911–920.

14 Bianchi G, Banfi A, Mastrogiacomo M et al. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res 2003;287:98–105.

15 Abbott A. Cell culture: biology's new dimension. Nature 2003;424:870–872.

16 Jacks T, Weinberg RA. Taking the study of cancer cell survival to a new dimension. Cell 2002;111:923–925.

17 Bancroft GN, Sikavitsas VI, van den DJ et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A 2002;99:12600–12605.

18 Wendt D, Marsano A, Jakob M et al. Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. Biotechnol Bioeng 2003;84:205–214.

19 Frank O, Heim M, Jakob M et al. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem 2002;85:737–746.

20 Baksh D, Davies JE, Zandstra PW. Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion-independent survival and expansion. Exp Hematol 2003;31:723–732.

21 Martin I, Mastrogiacomo M, De Leo G et al. Fluorescence microscopy imaging of bone for automated histomorphometry. Tissue Eng 2002;8:847–852.

22 Wodnar-Filipowicz A, Tichelli A, Zsebo KM et al. Stem cell factor stimulates the in vitro growth of bone marrow cells from aplastic anemia patients. Blood 1992;79:3196–3202.

23 Bianco P, Riminucci M, Gronthos S et al. Bone marrow stromal stem cells: nature, biology, and potential applications. STEM CELLS 2001;19:180–192.

24 Heng BC, Cao T, Stanton LW et al. Strategies for directing the differentiation of stem cells into the osteogenic lineage in vitro. J Bone Miner Res 2004;19:1379–1394.

25 Kale S, Biermann S, Edwards C et al. Three-dimensional cellular development is essential for ex vivo formation of human bone. Nat Biotechnol 2000;18:954–958.

26 Kapur S, Baylink DJ, Lau KH. Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways. Bone 2003;32:241–251.

27 Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. J Bone Joint Surg Am 1997;79:1699–1709.

28 Gronthos S, Zannettino AC, Graves SE et al. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J Bone Miner Res 1999;14:47–56.

29 Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 1995;85:929–940.

30 Quirici N, Soligo D, Bossolasco P et al. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 2002;30:783–791.

31 Friedenstein AJ, Latzinik NV, Gorskaya Y et al. Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner 1992;18:199–213.

32 Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 1977;91:335–344.

33 Wang Y, Uemura T, Dong J et al. Application of perfusion culture system improves in vitro and in vivo osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. Tissue Eng 2003;9:1205–1214.

Three-Dimensional Perfusion Culture of Human Bone Marrow Cells and Generation of Osteoinductive Grafts

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